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Research Note—

Impact of Different Husbandry Conditions on Contact and Airborne Transmission of H5N1 Highly Pathogenic Avian Influenza Virus to Chickens

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SUMMARY. Typically highly pathogenic avian influenza (HPAI) viruses spread very rapidly among chickens within sheds. However, the spread was slower than expected for the initial 10 days of the index farm in Japan during 2004. This slow spread, as well as the lack of gross lesions, clinical signs, or high mortality, hindered the field veterinarian from reporting a suspected HPAI outbreak to the veterinary office. To understand the field conditions for the slow virus spread, we examined contact and airborne transmission of the H5N1 virus to chickens in a negative-pressure isolator using various numbers of infected chickens and separate compartments. We found that the contact transmission did occur inefficiently when one or two chickens were infected, whereas the transmission was efficient when four chickens were infected. Airborne transmission of the HPAI virus was also dependent on the number of infected chickens and was less efficient than contact transmission. These data together with field observations suggested that number of infected chickens, chicken house types, and amount of environmental contamination might affect the virus transmission efficiency to chickens.

RESUMEN. *Nota de Investigación*—Impacto de diferentes condiciones de manejo en la transmisión por contacto y por aerosol del virus H5N1 de influenza aviar de alta patogenicidad en pollos.

Típicamente, los virus de influenza aviar de alta patogenicidad se diseminan rápidamente en los pollos dentro de la misma caseta. Sin embargo, la diseminación fue más lenta de lo esperado durante los primeros 10 días en la granja donde se presentó el problema inicial en Japón en el año 2004. Esta diseminación lenta junto con la ausencia de lesiones macroscópicas, signos clínicos o alta mortalidad, dificultó que el Veterinario de campo reportara un brote sospechoso de influenza aviar de alta patogenicidad a la agencia estatal. Para entender las condiciones de campo que explicaran la diseminación lenta, examinamos la transmisión por contacto y por aerosol del virus H5N1 para los pollos en una unidad de aislamiento mantenida a presión negativa, usando un número variable de pollos infectados y diferentes compartimientos separados. Encontramos que la transmisión por contacto ocurrió ineficientemente cuando uno o dos pollos estaban infectados, mientras que la transmisión fue eficiente cuando habían cuatro pollos infectados. La transmisión por aerosol del virus de influenza de alta patogenicidad también dependió del número de pollos infectados y fue menos eficiente que la transmisión por contacto. Estos datos junto con las observaciones de campo, sugieren que el número de pollos infectados, el tipo de caseta, y la cantidad de contaminación ambiental puede afectar la eficiencia de la transmisión del virus a los pollos.

Key words: avian influenza, H5N1, transmission

Abbreviations: AGP = agar gel precipitation; BID_{50} = 50% bird infective dose; dpi = days postinoculation; EID_{50} = 50% chicken embryo infective dose; HPAI = highly pathogenic avian influenza; LPAI = low pathogenic avian influenza; SPF = specific pathogen free

The H5N1 highly pathogenic avian influenza (HPAI) virus has been a great concern not only for the poultry industry but also for human health since 1997 (9). The virus has become endemic in poultry in East Asia since 2003, and as of 2006 it has spread to Western Asia, Europe, and Africa (the World Organization for Animal Health website: http://www.oie.int/eng/en_index.htm). The H5N1 virus has now spread to some wild bird populations (3), which may play an important role in the long-distance spreading of the virus.

The H5N1 HPAI virus Japanese strain spread slowly during the initial stage of the index farm (first Yamaguchi outbreak) in Japan between December 2003 and January 2004 (7), but based on the testimony of the farmers, the spread was fast during the Kyoto outbreak (third case) during February 2004. Since both H5N1 virus strains were highly pathogenic to chickens and genetically close (6), the transmission efficiency to chickens should have been similar. In addition to the slow spread in the Yamaguchi outbreak, the chickens lacked typical clinical signs or gross lesions of HPAI, which hindered the field veterinarian from quickly reporting the disease to an official veterinary office. The mechanism for the slow virus spread remains to be determined.

In this study, to better understand the conditions of the slow virus spread in the Yamaguchi outbreak, we tested transmission efficiency of the H5N1 virus from infected chickens to susceptible chickens in contact or through aerosol exposure using different numbers of infected chickens.

MATERIALS AND METHODS

Virus. The H5N1 HPAI virus, A/chicken/Yamaguchi/7/04 (Ck/Yama/7/04), isolated from a chicken from the index farm in Yamaguchi Prefecture, was used in this study (6). The virus was propagated in 10-

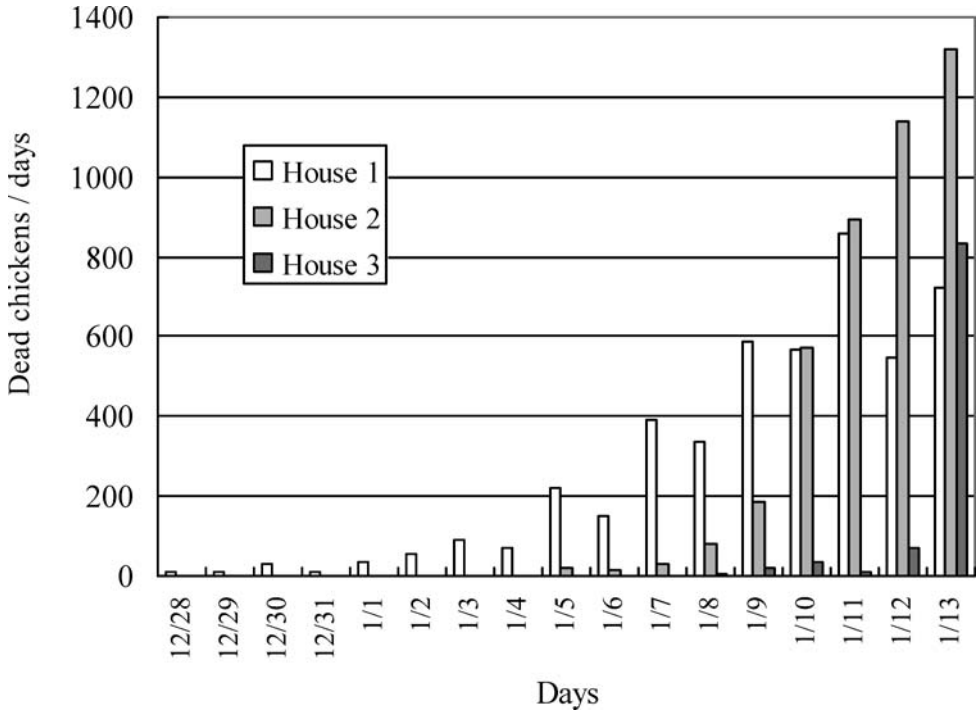

Fig. 1. Increase in dead chickens in three chicken houses in the Yamaguchi outbreak. A total of about 6000 layers were present in each chicken house.

day-old specific-pathogen-free (SPF) chicken eggs, and the allantoic fluid was harvested 2 days after inoculation and stored at −80 C before use under standard procedures (10). The fluid contained $10^{7.0}$ 50% chicken embryo infective dose (EID_{50})/0.1 ml.

Chickens. SPF chickens (4–6 wk of age) of line PDL-1 maintained in our institute were used (4). The SPF chickens were reared in a negative-pressure isolator in a negative-pressure chicken house (BSL 3). The isolator was subdivided into two compartments (compartments A and B, approximately 60 cm long, 70 cm wide, and 60 cm high) separated by two stainless steel wire nets 10 cm apart. Compartment A was used for intranasally inoculated and contact chickens to assess contact transmission. Compartment B was used to determine the airborne transmission from infected chickens in compartment A. Each compartment had its own feed and water supply. To maintain the chance of virus transmission, corrugated paper was set on a mesh floor in each compartment to prevent dropping of feces to the isolator floor. Dead chickens were left in the isolator until the end of the experiment to maintain the virus transmission condition from the dead chickens to susceptible chickens because dead chickens might be left in chicken houses for a while without notification. Leaving dead chickens in the isolator for the duration of the experiment was allowed by the Animal Ethics Committee of our institute.

Experimental designs. Four experiments were designed as follows.

Experiment 1. Nine chickens were separated into groups of one inoculated, four contact, and four separated chickens. The inoculated chicken was inoculated intranasally with 0.1 ml diluted allantoic fluid containing $10^{7.0}$ EID_{50} of Ck/Yama/7/04 and placed in compartment A with four contact chickens. Four separated chickens were placed in compartment B. These chickens were observed daily for 7 days for clinical signs and mortality.

Experiment 2. Eight SPF chickens were separated into groups of two inoculated, two contact, and four separated chickens. These chickens were placed as described above and observed daily for 7 days for clinical signs and mortality.

Experiment 3. Twelve chickens were separated into groups of four inoculated, four contact, and four separated chickens and placed as described above and observed daily for 10 days for clinical signs and mortality.

Experiment 4. To determine the chicken infective dose of Ck/Yama/7/04, 0.1 ml of 10-fold virus dilution ($10^{0.5}$ to $10^{4.5}$ EID_{50}/0.1 ml) were inoculated each of four 5-wk-old SPF chickens intranasally (five groups). These chickens were reared in each isolator and observed daily for 14 days for clinical signs and mortality. Surviving chickens were tested for the presence of agar gel precipitation (AGP) antibody to avian influenza virus using standard procedures (10). The chicken infective dose was calculated by the method of Reed and Muench (8).

RESULTS

Field outbreaks. In the first case in Yamaguchi Prefecture, the increase of mortality was slow for the initial 8 days. The chicken houses were classic, open-type, one-floor two-stage houses (6000 layers in each cage per house). On December 28, eight dead chickens were found at two sites near the windows at the entrance of chicken house 1, and mortality spread concentrically from these two sites. Mortality of chickens in chicken house 1 did not increase typically for the initial 8 days. On January 5th, the mortality had reached more than 200 chickens a day (Fig. 1). After that the virus spread quite fast, and 70% of the chickens in the house were dead before culling (January 13th). On the contrary, the H5N1 virus had furiously spread in chicken houses 2 and 3; the mortality rates had reached approximately 100 chickens per day 5 days after the onset (Fig. 1). The slow increase of chicken deaths for the first 7 days, in addition to the lack of typical respiratory signs or gross lesions, hindered the veterinarian from reporting the outbreak to the local government office.

Experiment 1. One day postinoculation (dpi), the chicken inoculated with the H5N1 virus showed ruffled feathers and slight depression but no respiratory signs, and then the chicken was dead at 2 dpi. However, four contact chickens in compartment A and four separated chickens in compartment B did not die over the 7-day observation period (Table 1).

Table 1. Experimental transmission of H5N1 highly pathogenic avian influenza virus to chickens.[A]

	Inoculated			Contact			Separate		
Experiment	Total	No. died	dpi of deaths	Total	No. died	dpi of deaths	Total	No. died	dpi of deaths
1	1	1	2	4	0		4	0	
2	2	2	2, 2	2	1	3	4	0	
3	4	4	2, 2, 2, 2	4	4	3, 3, 4, 4	4	3	4, 4, 8

[A]The isolator was divided into two compartments, A and B, by two wire nets 10 cm apart. Chickens inoculated intranasally with $10^{7.0}$ EID_{50}/0.1 ml of Ck/Yama/7/04 (H5N1) were reared with contact chickens in compartment A. Separated chickens in compartment B were used to determine the airborne transmission from infected chickens in compartment A.

Experiment 2. Since the result of Experiment 1 was unexpected, we repeated the experiment using two inoculated, two contact, and four separated chickens in an isolator. One of two inoculated chickens showed ruffled feathers and depression 1 dpi, and the two inoculated chickens were dead 2 dpi. One contact chicken showed depression in the morning 3 dpi and was dead in the afternoon. But one contact and four separated chickens were alive (Table 1). The surviving one contact and four separated chickens did not have AGP antibodies to avian influenza virus. These results demonstrated that contact transmission of the H5N1 HPAI virus was restrictive in some conditions, and airborne transmission was inefficient.

Experiment 3. To further understand the H5N1 transmission conditions, we used four inoculated, four contact, and four separated chickens as described above. One of the inoculated chickens showed ruffled feathers and depression 1 dpi, and four inoculated chickens died 2 dpi (Table 1). Two contact chickens died 3 dpi, and the other two contact chickens showed depression 3 dpi and then died 4 dpi (Table 1). In addition, two separated chickens showed depression 3 dpi and died 4 dpi. One separated chicken died 8 dpi, while one separated chicken survived for 10 days. These results indicated that contact and airborne transmission occurred efficiently when four chickens were infected. Nevertheless, surprisingly, one separated chicken survived for 10 days without infection. No AGP antibody, histopathologic lesions, or virus recovery were detected in the surviving chicken.

Experiment 4. All of the chickens inoculated with $10^{2.5}$ to $10^{4.5}$ EID_{50} were dead within 4 days, but chickens inoculated with $10^{1.5}$ to $10^{0.5}$ EID_{50} survived. The surviving chickens did not have any AGP antibody. Therefore, the 50% chicken infective dose of Ck/Yama/7/04 was $10^{2.5}$ EID_{50}, which was equal to the chicken lethal dose, since all infected chickens died.

DISCUSSION

The H5N1 Ck/Yama/7/04 strain was highly pathogenic and well adapted to chickens (5) with a 50% chicken infective dose of $10^{2.5}$ EID_{50} (Experiment 4). Therefore, it seems likely that the H5N1 virus could spread quickly in chicken populations. Nevertheless, in Yamaguchi outbreak, the virus spread slowly in the first chicken house for the initial 8 days but spread quickly after that followed by houses 2 and 3 (Fig. 1). In this study, contact and airborne transmission of the virus was shown to be less efficient when one or two chickens were infected with the H5N1 Ck/Yama/7/04 strain, whereas the transmission was efficient when four chickens were infected. These data could partly explain the reason why the H5N1 virus spread slowly at the initial stage of the Yamaguchi outbreak.

Transmission of avian influenza virus from intranasally infected chickens to chickens placed in contact varied considerably among six HPAI and two low pathogenic avian influenza (LPAI) virus strains, with the most efficient transmission occurring with two HPAI virus strains (1). Our study showed that even if the floor was covered with corrugated paper and dead chickens were kept in the isolator until the end of the experiment, one of two contact chickens in Experiment 2 and one of four separated chickens in Experiment 3 survived (Table 1). We confirmed that the virus titers recovered from tracheal and cloacal swabs of dead chickens were $10^{3.5}$ to $10^{5.0}$ EID_{50} and $10^{2.0}$ to $10^{5.0}$ EID_{50}, respectively (data not shown). Therefore, one possible explanation for the survival of the chickens is that the surviving chickens might have had insufficient exposure to virus shed into the environment, or the infected chickens might have died quickly before discharging significant amounts of virus before death, thus making exposure less than the 50% chicken infective dose of $10^{2.5}$ EID_{50} of virus. Prior studies with H7N2 LPAI virus demonstrated 50% bird infective doses (BID_{50}) of $10^{0.8}$, $10^{2.8}$, and $10^{3.2}$ EID_{50} for SPF turkeys, white leghorn, and white Plymouth Rock chickens, respectively (11). These differences in BID_{50} may be an explanation for virus strain differences in host susceptibility and the greater number of infected farms housing turkeys compared with chickens.

Another contributing factor for slow virus transmission may have been the type of chicken houses. In the Kyoto outbreak, the actual mortality record was not known because the record was discarded by the owner to conceal the outbreak. According to the testimony of the workers, the H5N1 Kyoto strain probably spread quickly from the initial stage. It was shown that the A/chicken/Kyoto/3/04 was quite similar genetically to the Ck/Yama/7/04 strain, and highly pathogenic to chickens (6). In the Kyoto outbreak, the death of a group of chickens was confirmed on February 17 in the chicken house, and on next day about 100 dead chickens were found in the same rows of cages where the original outbreak occurred, and then from the 20th, the death had spread throughout the entire house with more than 1000 deaths a day. Most of the chickens in the house were dead within 10 days. In contrast, elevated mortality was not seen for the initial 8 days in the Yamaguchi outbreak. We visited both outbreak farms to clarify the reasons for the difference in the virus transmission efficiency and found that the types of chicken houses were different. The chicken house of the Kyoto farm was open-type, elevated-floor, four-stage double type houses (30,000 layers/house), and there were big fans on the floor of the chicken house to dry droppings, which may have increased efficiency of airborne virus spread within the house. By contrast, the house of the Yamaguchi outbreak was a classic, open-type, one-floor two-stage house without fans, and the door was opened in the daytime (6000 layers/house). Therefore, it is possible that chicken house types may affect the virus transmission efficiency at the initial stage of outbreaks. Previously, transmission of H7N1 HPAI virus was more rapid in chickens housed on litter with freedom to move about than laying chickens housed in cages (2).

In conclusion, our study together with field observations suggested that the chicken house types, number of infected chickens,

and amount of environmental contamination may have affected the virus transmission efficiency during the H5N1 HPAI outbreak. Our study also suggested that continuous appearance of a small number of dead chickens at a spot for a few days should be immediately investigated for HPAI.

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